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THE ROLE OF THE ELECTROGENIC SODIUM PUMP IN MODULATION OF PACEMAKER DISCHARGE OF APLYSIA NEURONS

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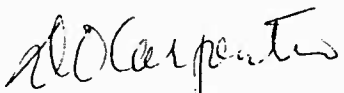

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ABSTRACT

The discharge of Aplysia pacemaker neurons varies with temperature over the range 10^{o} to 22^{o}C. Three types of temperature-frequency plots are found, with maximal discharge at lowest, intermediate or highest temperatures. In the presence of ouabain, however, all cells show maximal discharge at the highest temperature, suggesting that the steady-state activity of an electrogenic sodium pump is an important determinant of membrane excitability. The average magnitude of pump current, as indicated by the applied current necessary to restore discharge to control values after ouabain application, was about 4 nA at 20^{o}C but near zero at 10^{o}C. These neurons may be excellent models of mammalian thermoreceptors.

I. INTRODUCTION

Electrogenic sodium pumps have been demonstrated in a wide variety of nerve and muscle cells[25] but generally under conditions where the rate of sodium transport has been artificially increased by sodium loading. Since pump activity (and thus the electrogenic current) is directly proportional to intracellular sodium,[26] a greater current is generated if the cell is sodium loaded. This has been accomplished by tetanic stimulation of nerves, allowing sodium to enter through natural mechanisms,[19] by soaking muscle in the cold under conditions where transport is blocked and sodium accumulates[16] or by direct iontophoretic injection of sodium into a single neuron.[24] These studies have shown that an electrogenic pump can develop a considerable potential when stimulated but have not determined whether the pump contributes potential in steady-state conditions without sodium loading.

In Aplysia neurons the presence of an electrogenic pump is easily demonstrated without sodium loading by subjecting the isolated nervous tissue to rapid temperature changes.[7] On rapid warming, these neurons hyperpolarize. This hyperpolarization can be abolished by cardiac glycosides, such as ouabain, absence of K^+ or substitution of Li^+ for Na^+, all of which block activity of Na^+-K^+ adenosine triphosphatase, the enzyme mediating Na^+ transport. In silent nonpacemaker neurons the temperature-dependent potential shifts attributable to the electrogenic pump may be as much as 50 mV in some cells.[4] Although temperature-dependent potential shifts are present in pacemaker neurons as well, their magnitude is more difficult to measure due to the spontaneous pacemaker discharge. Moreover in none of these neurons has the steady-state role of the pump been satisfactorily studied, although it has been suggested[4] that

the large transient voltage shifts obtained on rapid temperature changes are evidence that the electrogenic Na^+ pump contributes to resting membrane potential.

Endogenous pacemaker activity is characteristic of many invertebrate neurons including the majority of the neurons in the abdominal ganglion of Aplysia.[3,11] This pacemaker activity originates in the cell body[1] unlike the synaptically initiated spikes which arise in the axon.[23] The pacemaker action potentials are usually dependent on the presence of external sodium but in those cells whose action potential is calcium dependent and sodium independent normal patterns of pacemaker activity may occur in the complete absence of external sodium as long as calcium is present in the medium.[8] The ionic mechanisms underlying this endogenous activity are poorly understood. However, it is likely that the pacemaker discharge results from a time- and voltage-dependent decrease in potassium permeability (P_{K^+}) following action potentials in the face of a relatively high resting sodium permeability (P_{Na^+}) (or perhaps calcium permeability).[5,15] This mechanism is identical to that postulated to explain the spontaneous activity of the Purkinje fibers of the heart.[27]

In these experiments we analyze the steady-state contribution of the electrogenic pump through its effect on pacemaker discharge. Because the frequency of pacemaker spikes is a direct function of depolarizing current applied across the membrane[5] and because the steady-state contribution of the pump is simply a current, we used the discharge frequency to measure the current generated by the pump. In addition we examined the interactions between the pump current and other temperature-dependent mechanisms underlying endogenous activity.

II. MATERIALS AND METHODS

Specimens of Aplysia californica were obtained from Pacific Biomarine Supply Company, Venice, California and maintained in artificial seawater at 15°C. The abdominal ganglion was removed, placed in an epoxy specimen chamber and perfused with artificial seawater. The temperature of the perfusate was controlled by a thermoelectric heat pump (Cambion Model 196) and monitored by a bead thermistor located in the specimen chamber. Cells were identified according to Frazier et al.[11] Identified cells were penetrated with either one or two 3 M KCl filled micropipettes. Intracellular potentials were measured with BAK A-4 electrometer amplifiers.

In experiments where cell parameters were measured as a function of temperature, the perfusate temperature was increased from 10° to 22°C in 2° increments. At least 10 minutes of data were recorded at each temperature to allow for accommodative changes. When constant temperatures were required, the cooling system maintained the desired temperature within ± 0.5°C for the duration of the experiment.

Pacemaker discharge frequency was determined by processing the intracellular potential recording with a pulse discrimination circuit to detect each action potential. The interval between successive action potentials was then recorded to the nearest 0.01 second with an identifying time code on digital magnetic tape. The mean firing frequency for any selected epoch of pacemaker activity could thus be calculated, using an SDS 920 computer.

In studies of temperature dependence of pacemaker activity, the cell was penetrated with a single microelectrode and at least two frequency versus temperature determinations were made on the control preparation. Following these determinations,

the preparation was incubated for 10 to 15 minutes in a 10^{-4} M ouabain-artificial seawater solution. After incubation, two additional frequency versus temperature determinations were made.

In studies where input resistance was measured, two micropipettes were inserted independently into the cell. One micropipette was used to pass 1.0 nA hyperpolarizing current pulses while the other micropipette recorded intracellular potential. Input resistance was measured in control and ouabain poisoned cells from 10^{o} to 22^{o}C.

Measurement of the magnitude of the electrogenic pump current was accomplished using the frequency of pacemaker discharge to indicate the net change in membrane current resulting from poisoning the electrogenic pump with ouabain. The preparation was maintained at a selected constant temperature throughout the experiment. A membrane current-firing frequency relationship was first derived by passing known depolarizing currents through the cell membrane while simultaneously recording pacemaker discharge frequency. Several minutes of data were recorded at each current setting to obtain postaccommodative firing rates. The discharge at 10 minutes after the start of current application was used as the adapted frequency at that potential. The sodium pump was poisoned by incubation in 10^{-4} M ouabain for 10 to 15 minutes. The change in pacemaker frequency was recorded, and the pump current calculated from the current versus frequency relationship. For bursting pacemaker neurons only the discharge during the burst was analyzed.

III. RESULTS

The pacemaker neurons of the abdominal ganglion of Aplysia can be divided into three distinct groups on the basis of the changes of endogenous discharge rate with changing temperature.

Of the 40 cells studied, 22 (55 percent) behaved as "warm" receptors, increasing their discharge frequency as the perfusion temperature was measured from 10^{o} to 22^{o}C (Figure 1A, control). Most cells exhibiting this behavior were the pigmented cells of the left hemiganglion, L2, L3, L4, L6, L8 and L11. Cells L7 and R15 appeared to behave in a similar fashion but, owing to the complex nature of their bursting activity and synaptic inputs, quantitative measurement of pacemaker discharge frequency was difficult. A second group of 14 cells exhibited a temperature response (Figure 1B, control) similar to that found in mammalian temperature receptors[10] with a maximal discharge at an intermediate temperature. The majority of these responses were found in the unpigmented cells of the upper right hemiganglion. Of these cells, R6 was most frequently studied. All R6's which behaved in this manner exhibited activity peaks between 16^{o} and 18^{o}C. The third type of response was that of a pure cold receptor, in which pacemaker discharge frequency decreased as temperature increased (Figure 1C, control). This response was found in three experiments in cell R6 and twice in cell R14. In the experiment illustrated, discharge was absent above 19^{o}C and increased on cooling. Figure 2 (controls) shows typical recordings from cold (A) and warm (B) sensitive neurons upon a relatively abrupt temperature change. The warm sensitive cell in Figure 2B has the bursting discharge characteristic of some identified cells.

Abolition of the electrogenic pump current by incubation in 10^{-4} M ouabain could be expected to remove any modulating effect of the pump on the temperature dependence of the pacemaker discharge. All control experiments described previously were followed by ouabain incubations and the effects of application of 10^{-4} M ouabain

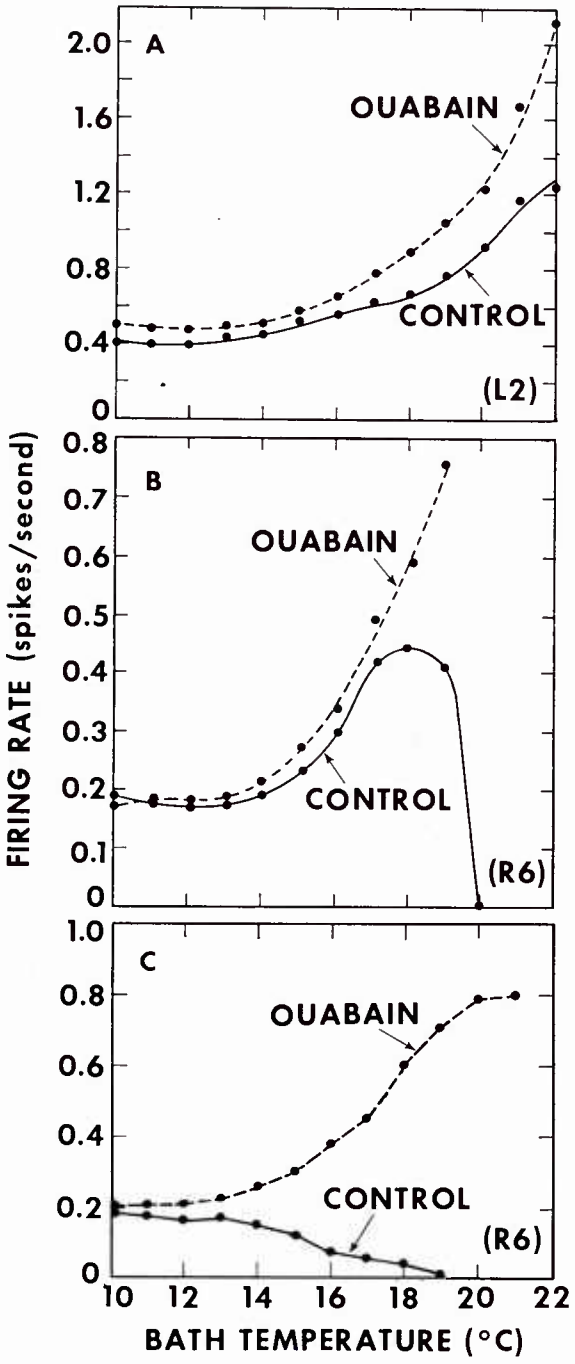

Figure 1. Adapted temperature-frequency plots for three different neurons before and after ouabain. A. Cell L2. B. and C. Separate experiments on cell R6. Each point represents average frequency over 2 minutes taken at least 10 minutes after the change of temperature. Ouabain responses were obtained following a 10-minute incubation with 10^{-4} M ouabain, after which ouabain was washed out before responses were recorded.

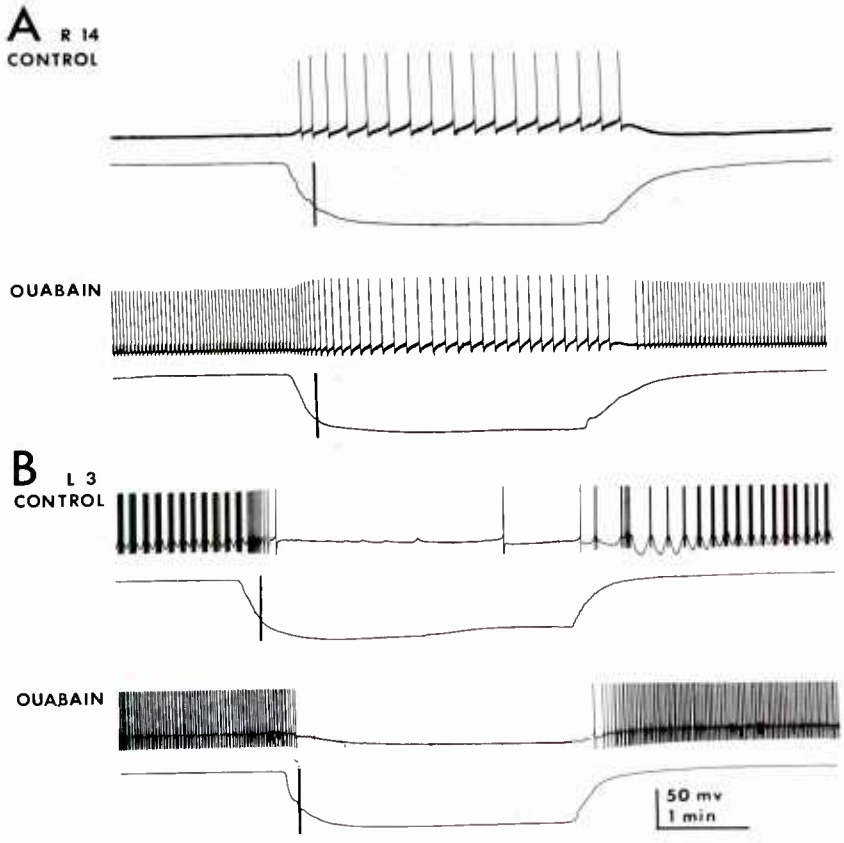

Figure 2. Discharge patterns on sudden temperature changes before and after ouabain in cells R14 and L3. In each case the upper trace is the intracellular recording, while the lower trace is temperature where the vertical bar indicates 5° and 20°C at lower and upper ends respectively. In R14 the response changes from that of a "cold receptor" to that of a "warm receptor" following 10^{-4} M ouabain for 10 minutes. Cell L3 in the control illustrates its normal bursting pattern. After ouabain, this pattern is altered and discharge is more regular.

for 10 minutes are illustrated in Figures 1 and 2. Cells exhibiting warm receptor responses increased their firing rate, and the cells' sensitivity as a warm receptor was enhanced. Cells exhibiting a peaked temperature response curve reverted to the warm receptor response. Many cells of this type which would cease firing above 20°C in the control situation responded with a smoothly increasing firing rate to beyond 24°C. Cells exhibiting a cold receptor response dramatically changed to warm receptor

response following ouabain. In all cases the discharge was not significantly changed by ouabain at 10^{o}C as compared to the control. After ouabain the general shape of the temperature-frequency plot was similar for all three types of pacemaker neurons.

The results indicate that an electrogenic sodium pump modulates pacemaker discharge in all endogenously active cells of Aplysia. Furthermore, the observation that after ouabain the temperature-frequency plots of all pacemakers show the same general form suggests that the pump, interacting with another temperature-dependent mechanism tending to increase discharge on warming, determines whether a given cell has the discharge pattern of a cold, peaked, or warm receptor.

To further analyze the interaction between the pump and the depolarizing force on warming (previously identified as a passive sodium permeability increase[4,12,13]), a series of experiments were performed on cell R6. This cell is large, easily identified and has almost no spontaneous synaptic inputs. Thus its spontaneous discharge is exclusively endogenous. Of the twenty-eight R6 cells in this series of the experiment, all but two showed a peaked temperature-frequency response. Other electrophysiologic parameters, such as spike amplitude and resting membrane potential, were also very similar among experiments.

Figure 3 shows the temperature dependence of total input resistance reflecting net ionic conductance in seven R6 cells in normal seawater and four cells after ouabain. Over the temperature range between 10^{o} to 20^{o}C, the input resistance decreased by about 20 percent. There is a large variability, reflecting probably both a real physiologic difference in this cell's resistance from preparation to preparation as well as differing degrees of injury of penetration. There is, however, no statistically

significant difference between control and ouabain-treated cells, indicating that operation of the electrogenic pump is not associated with a conductance change. Furthermore, there is no sudden change in resistance at or about 16^{o} - 18^{o}C which might explain the discharge peak seen at that temperature in most preparations.

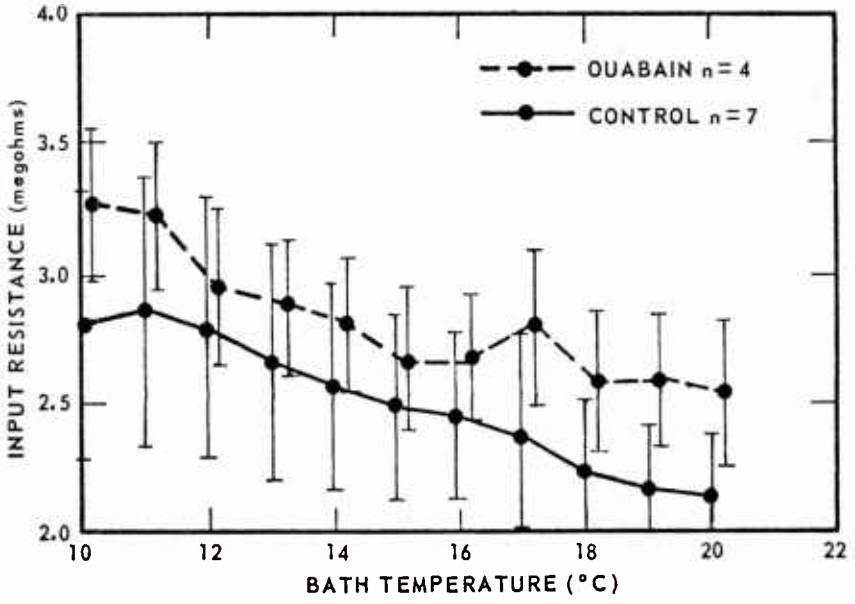

Figure 3. Average input resistance (with standard error) as a function of temperature in cell R6 before and after ouabain. There is no significant effect of ouabain.

In order to determine the magnitude and the temperature dependence of the current generated by the electrogenic pump, experiments were performed using the frequency of discharge as a monitor of cell membrane potential. As shown by Carpenter,[5] pacemaker discharge in these neurons is a linear function of applied current, with discharge increasing on depolarization and decreasing on hyperpolarization. Since the electrogenic pump is essentially a hyperpolarizing current source, its removal by

ouabain results in both a depolarization and an increased discharge. The degree of depolarization cannot be measured directly in these pacemaker cells because of the discharge in contrast to the situation in silent cells. However, if the relationship between frequency and current is known in the control period, the change in frequency caused by ouabain can be equated to the removal of a corresponding hyperpolarizing current. One such experiment is shown in Figure 4. The solid line represents the frequency-current relationship in the control. After ouabain the frequency rose from 0.3/sec to 1/sec. This frequency corresponded to that obtained by applying about 10 nA in the control. Thus in this cell the total ouabain-sensitive current at this temperature is 10 nA, assuming that a negligible amount of the applied current flows down the axon.

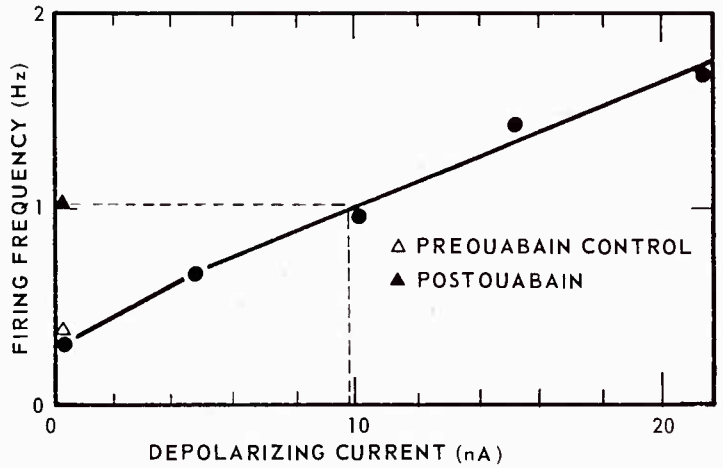

Figure 4. Determination of pump current in cell R6. Solid line shows firing frequency as a function of applied depolarizing current in normal seawater. All points were obtained as 2-minute averages of frequency at least 10 minutes after initiation of current passage. The triangle shows frequency 10 minutes after ouabain application and the dashed lines indicate that in this cell the discharge frequency in ouabain is identical to that produced in the control by passage of about 10 nA. Experiment was performed at 20°C.

The magnitude of electrogenic pump current was determined for twenty-one R6 cells. Due to the irreversible action of ouabain in Aplysia, this determination could be made only once in each cell. Thus experiments were performed in three cells at each of seven temperatures from 10^{o} to 22^{o}C. The average ouabain-sensitive current (plus standard errors) is shown in Figure 5. Between 10^{o} and 20^{o}C, the pump current rose from 0.3 to 4 nA, an increase of about tenfold. The rate of increase of pump current with temperature was greatest in the range between 12^{o} and 16^{o}C.

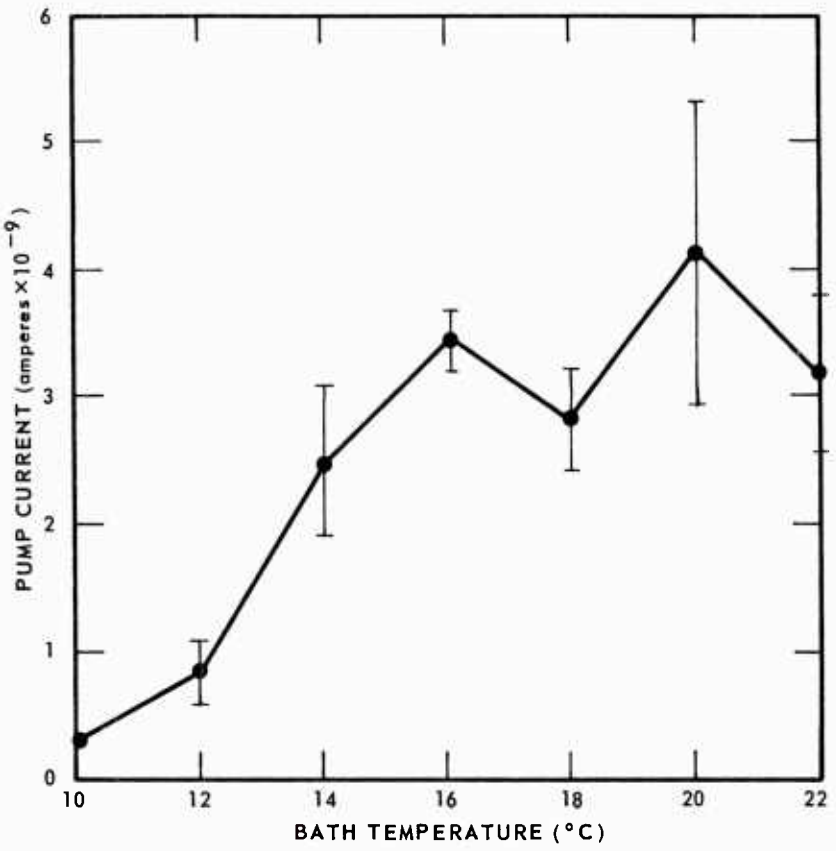

Figure 5. Average pump current (and standard error) as measured in Figure 4 as a function of temperature from twenty-one R6 cells

Table I gives similar measurements on other identified pacemaker neurons at various temperatures. For none of these cells are the data sufficient to plot as in Figure 5, but the results appear very consistent with those obtained for cell R6.

Table I. Ouabain-Sensitive Pump Currents at Various Temperatures in Identified Neurons

Cell	Temperature (^{o}C)	Pump Current (x 10^{-9} amperes)
L2	12	2.2
L2	15	4.0
L2	19	8.2
L6	12	2.0
L6	20	4.6
L6	20	11.0
L6	22	3.7
L6	22	4.4
L6	22	5.0
L6	22	7.0
R14	14.5	1.0
R4	15	4.0
L3	19	8.7
R15	20	9.0
R15	22	13.0

IV. DISCUSSION

The steady-state pump current. These experiments show that an electrogenic Na^+ pump generates an average steady-state current of about 4 nA at 20^{o}C in Aplysia pacemaker neurons. This current is sufficient to exert clear effects on the cell excitability, and thus we conclude that the steady-state activity of an electrogenic pump has physiological significance in these neurons. The neuron could regulate its endogenous discharge by controlling pump rate, the coupling ratio of Na^+ to K^+ pumped,[17] or

through metabolic regulation of membrane resistance, thus changing the voltage generated. Since the electrogenic current flows across the membrane resistance to produce a voltage, the effect on discharge frequency is proportional to both pump current and resistance. It is known that the membrane resistance of these neurons is extraordinarily high (at least 100,000 ohm cm^2)[4] as compared to many other excitable tissues and that this resistance falls dramatically in the presence of metabolic inhibitors.[9] Metabolic regulation of resistance in the face of a relatively constant electrogenic current might be a basis for the circadian and other rhythms described in some of these pacemaker neurons.[21]

In a neuron with an input resistance of 2.5×10^6 ohms, a 4.0-nA current is equivalent to a voltage of 10 mV. This value is not so high as reported for one experiment on the nonpacemaker cell R2[4] but was obtained as the average from a number of neurons and is probably much more representative of the pump currents of both pacemaker and nonpacemaker cells. A similar average pump-dependent voltage has been measured in Anisodoris neurons[12] and in cell bodies of the stellate ganglion of the squid.[6]

It is possible that the Na^+ pump was not totally inhibited in these experiments. Because prolonged exposure to ouabain may sometimes lead to a ouabain-caused increase in membrane conductance,[5] the duration of the exposure was limited to 15 minutes (such ouabain-induced conductance changes did not occur in these experiments, as shown in Figure 3). Since the pump may not have been totally inhibited, the 4-nA average pump current should be viewed as a minimal value.

The nature of the passive mechanisms responsible for increased discharge on warming. In the presence of ouabain, all pacemaker neurons showed an increasing

discharge as temperature rises. This effect was attributed by Carpenter[4] to a greater temperature coefficient of P_{Na^+} than of P_{K^+}. This study showed that lowering external Na^+ with substitution of an impermeant cation abolished the depolarization on warming although, even in the absence of Na^+, membrane resistance fell slightly on warming, reflecting the temperature coefficient of P_{K^+}. Gorman and Marmor[12] performed a more rigorous study on a similar effect in Anisodoris neurons and demonstrated that in the presence of ouabain membrane potential was proportional to the P_{Na^+}/P_{K^+} ratio. Since the membrane depolarized on warming, this indicates that P_{Na^+} changes more than P_{K^+}, thus moving the potential toward the equilibrium potential for Na^+. In squid axon there is also a greater Q_{10} for P_{Na^+} (1.4) than for P_{K^+} (1.1).[14]

In all experiments the discharge frequency at 10^{o}C did not change significantly on ouabain application. This observation is important for two reasons. It indicates that the pump does not generate appreciable current at this temperature, and thus that the transport process has a very high temperature coefficient. Also it indicates that treatment with ouabain for 15 minutes does not functionally change the intracellular ionic concentrations. Gorman and Marmor,[12] in a closely related preparation, have shown that membrane potential at low temperatures is adequately described by passive membrane permeabilities and the constant field equation. At higher temperatures the constant field equation no longer described the potential, due to operation of the pump. If intracellular ionic concentration has significantly changed, we would expect this to be reflected in a potential change.

Over a moderate range of currents, the endogenous discharge of these neurons varies linearly with applied transmembrane current (Figure 1 in Carpenter[5]). Thus

the discharge frequency in the presence of ouabain reflects the changing P_{Na^+}/P_{K^+} ratio. The observation that all neurons showed a smooth increase in discharge with temperature after ouabain suggests that the Q_{10} values for P_{Na^+} and P_{K^+} are similar in all these cells.

Both the capacity of these neurons to fire endogenously and the ability of the electrogenic pump to influence the discharge so dramatically in a steady-state situation result from the high membrane resistance. The high resistance relative to other nerve and muscle cells reflects a low P_{K^+}, and thus the P_{Na^+}/P_{K^+} ratio is smaller than in other tissues. As demonstrated by Trautwein and Kassebaum[27] pacemaker discharge results from a time and voltage dependence of P_{K^+} in the presence of a depolarizing force which is P_{Na^+}. In this respect, it is likely that both the presence of endogenous discharge and the occurrence of a large electrogenic pump potential are indicative of cells with high membrane resistances.

Interaction between passive and metabolic mechanisms. All three types of temperature-frequency responses can be explained on the basis of the interaction between the opposing effects of the temperature dependence of the P_{Na^+}/P_{K^+} ratio and the electrogenic pump current. That both mechanisms exist in all these neurons is indicated by the fact that ouabain affects all cells, especially at higher temperature, and by the similarity of responses in all neurons after ouabain. In the warm sensitive neurons a pump current modulates the passive effects, but the change in P_{Na^+}/P_{K^+} ratio with temperature predominates over the whole temperature range. In contrast in the cold sensitive neurons the pump current predominates at all temperatures even though passive effects like those observed in the other cells appear when the pump is

blocked. In those neurons where there is an intermediate temperature where frequency is maximal, the passive mechanisms predominate at temperatures below peak discharge, but the pump current predominates at higher temperatures. It is not surprising that a sizable percentage of cells show this response since, whereas total resistance changed by a factor of two between 10^{o} and 20^{o}C, the pump current changed by a factor of 10. The Q_{10} of input resistance does not, of course, reflect the Q_{10} of the P_{Na^+}/P_{K^+} ratio, but the coefficient for the ratio is probably not very different.

The electrogenic Na^+ pump thus does act to modulate endogenous pacemaker discharge in Aplysia pacemaker neurons, regardless of the form of their temperature-frequency relations. It is unlikely, however, that the pump is responsible for other influences on pattern of discharge. It has been suggested by Ayrapetyan,[2] Romey and Arvanitaki-Chalazonitis[20] and Strumwasser[22] that an oscillatory electrogenic Na^+ pump is responsible for the bursting patterns seen in some neurons as in Figure 2. However, these patterns are not ouabain sensitive, although they are changed by polarization.[5]

These neurons are excellent models for specific temperature sensitive afferent fibers of higher animals. Most mammalian temperature sensitive afferents show an optimal temperature with a falling discharge at either side of the optimal temperature.[10] As with the Aplysia neurons of this type, this pattern may reflect the interaction between the passive depolarizing effect of a P_{Na^+}/P_{K^+} ratio which increases with temperature and has a relatively low Q_{10} and the hyperpolarizing influence of an electrogenic Na^+ pump, dependent upon rate of transport and the magnitude of membrane resistance, which has a high temperature dependence. Support for these

mechanisms in specific rat cold receptors and cold sensitive mechanoreceptors has recently been obtained by Pierau et al.[18] who showed that these cold sensitive neurons became warm sensitive upon exposure to ouabain.

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13. ABSTRACT

The discharge of Aplysia pacemaker neurons varies with temperature over the range 10° to 22°C. Three types of temperature-frequency plots are found, with maximal discharge at lowest, intermediate or highest temperatures. In the presence of ouabain, however, all cells show maximal discharge at the highest temperature, suggesting that the steady-state activity of an electrogenic sodium pump is an important determinant of membrane excitability. The average magnitude of pump current, as indicated by the applied current necessary to restore discharge to control values after ouabain application, was about 4 nA at 20°C but near zero at 10°C. These neurons may be excellent models of mammalian thermoreceptors.

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